FINAL PROJECT REPORT

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I INTRODUCTION

The finding (1) that thioctic acid (ThA)[1] is removed from extracts of the oiliated protozoan, Tetrahymena pyriformis, by treatment with adsorption alumina suggested that this procedure may provide a tool for investigations of functions of ThA other than the established participation in α-keto acid oxidations (2, 3). The role of the cofactor in these oxidations suggests participation in additional acyl transfer reactions. The animal acetate-activating system (reaction 1) seemed a likely suspect.

$$\text{acetate} + \text{ATP} + \text{Co A} \rightarrow \text{acetyl-Co A} + \text{AMP} + \text{PP} \qquad (1)$$

II RESULTS

Thioctic acid requirement of the acetate system

Alumina treatment of pigeon liver acetone powder extracts decreases the rate of acetyl-Co A formation from acetate; the thioctic acid content of the extract is also decreased (Table I). Addition of synthetic thioctic acid restores activity to mixtures containing treated enzyme.

TABLE I. Alumina Treatment of Acetone Powder Extracts

System 1 contained in 1.0 ml: 75 μM Na acetate; 20 units Co A; 100 uM Tris buffer, pH 8.2; 10 μM GSH; 200 uM hydroxylamine; 10 μM Na-ATP; 10 uM $MgCl_2$; 60 μM NaF. System 2 contained in 1.0 ml: 70 μM Na acetate; 20 μM Co A; 100 μM phosphate buffer, pH 8.2; 10 μM GSH; 10 μM Na-ATP; 10 μM $MgCl_2$; 60 μM NaF; S. faecalis extract containing 0.3 μg protein. System 3 contained in 1.0 ml: 75 μM Na acetate; 20 units Co A; 100 μM Na-ATP; 10 μM $MgCl_2$; 60 μM NaF. In all cases 18 mg protein of extract were incubated 90 min. at 32° C. The ThA content of the extract added was 0.527 μg. After alumina treatment the cofactor content was 0.05 ug.

Enzyme treatment and additions	System 1 hydroxamate formed	System 2 acetyl phosphate formed	System 3 citrate formed
	μM	μM	μM
None	1.23	1.17	1.20
+ 0.1 μg ThA	1.19	1.15	1.21
Alumina treated	0.11	0.20	0.16
+ 0.1 μg ThA	1.17	1.16	1.16

1. The following abbreviations are used: ATP, adenosine triphosphate; AMP, adenosine monophosphate; PP, inorganic pyrophosphate; Co A, coenzyme A; BAL, 2,3-dimercaptophropanol; GSH, glutathione; Tris, tris-(hydroxymethyl)-aminomethane; ThA, thioctic acid; DPN, diphosphopyridine nucleotide; DPT, diphosphothiamine; Pi, inorganic phosphorus.

As anticipated in a ThA dependent system (4), arsenite inhibits the acetate-activating reaction; arsenate is without effect. Adjustment of the thioctic acid content of extracts to just optimal levels by alumina treatment and the addition of synthetic thioctic acid results in preparations which are much more sensitive to the inhibitor than are untreated extracts which contain excess cofactor. The arsenite inhibition is reversed by BAL, but not by such monothiol compounds as cysteine, thioglycollate, or additional amounts of GSH (Table II).

TABLE II. Arsenite Inhibition of Acetate Activating System

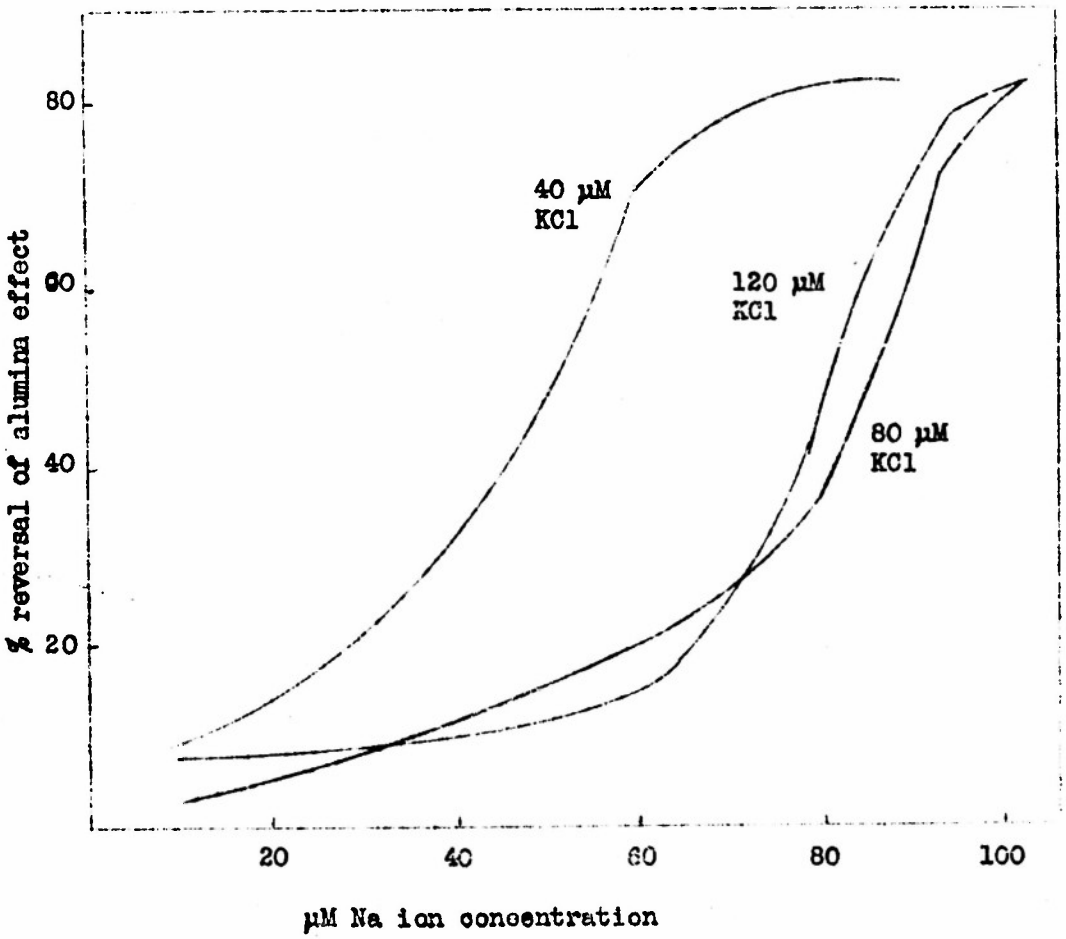
The incubation mixture was as described in System 1 of Table I. The extract containing 15 mg. of protein was incubated for 90 min. at 32° C. The ThA content of this amount of untreated extract was 0.466 µg. After alumina treatment the cofactor content was 0.053 µg.

Additions	Hydroxamate formed	
	Untreated extract	Alumina treated extract + 0.1 µg ThA
	µM	µM
None	0.92	0.84
2 µM arsenite	0.88	0.51
4 µM arsenite	0.71	0.24
8 µM arsenite	0.62	0.08
8 µM arsenate	0.91	0.81
4 µM arsenite + 20 µM cysteine	0.68	0.26
4 µM arsenite + 20 µM thioglycollate	0.70	0.23
4 µM arsenite + 10 µM additional GSH	0.66	0.25
4 µM arsenite + 5 µM BAL	0.90	0.79

Reversal of the alumina effect by added ThA is stimulated by sodium ions and is inhibited by potassium ions (Fig. 1). However, in the presence of high

FIGURE 1. Sodium Ion Stimulation of ThA Reversal of Alumina Treated Acetate Enzyme at various levels of Potassium Ion Concentration

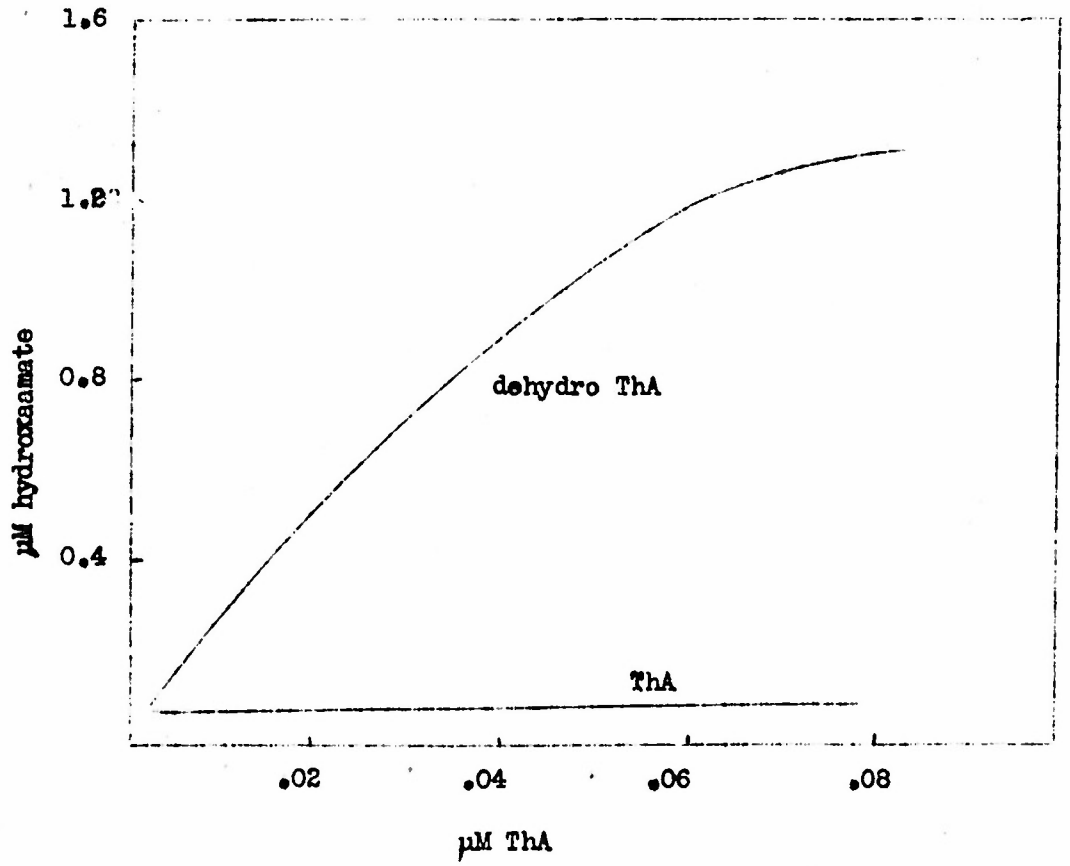
The incubation mixtures were as described for System 3 in the legend of Table I. All acidic components were converted to their Tris salts. Pigeon liver acetone powder was extracted with 0.1 M Tris buffer, pH 8.2. Activity before alumina treatment in each of the various mixtures was taken as 100%. ThA (0.1 µg) was included in mixtures after alumina treatment. KCl and NaCl were added as indicated.



concentrations of potassium ions, where ThA is inactive, the reduced form of the cofactor, dihydrothioctic acid, increases the rate of acetyl-Co A formation (Fig. 2). For activity in the acetate activating reaction then, the disulfide linkage of ThA must be reductively split by a system which is stimulated by sodium ions and inhibited by potassium ions. This system is inactivated by repeated freezing and thawing.

FIGURE 2. Stimulation of Alumina Treated Extracts by Dihydrothioctic Acid in the Presence of Large Amounts of Potassium Ion

The incubation mixture was as described for system 1 in the legend of Table I, except that 100 μM of KCl were included. The extract (18 mg of protein) was alumina treated and incubated 90 min. at 32° C.



The 8-methyl derivative of ThA which is antagonistic to the cofactor for the growth of several microorganisms (5), also inhibits the acetate activating

reaction; the inhibition is reversed by ThA (Fig. 3). In the presence of high concentrations of potassium ion the analog is not inhibitory (Table III). To be

FIGURE 3. Effect of 8-methyl ThA on Acetate Activating Activity of Acetone Powder Extracts at Various Levels of ThA

The incubation mixture was as described for system 1 in the legend of Table I. Fifteen mg of protein containing 0.533 µg of thioctic acid were incubated for 90 min. at 32° C.

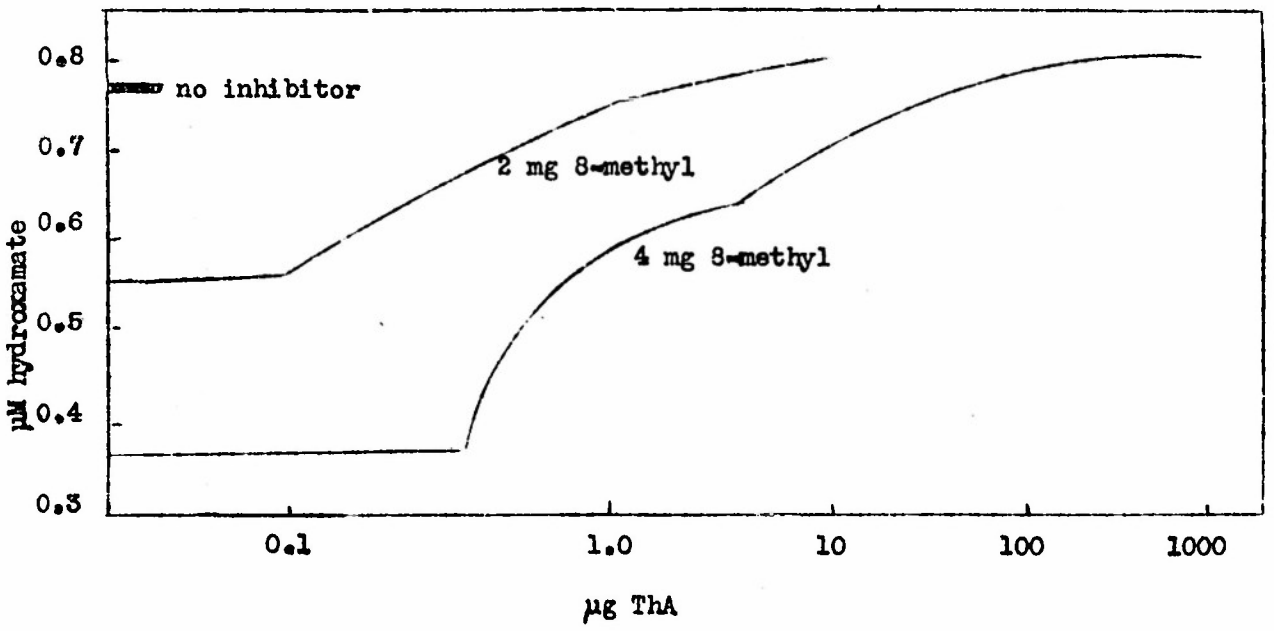


TABLE III. Effect of Potassium Ions on 8-methyl ThA Inhibition of Acetate Activation

The incubation was a described for system 2 in the legend of Table I. KCl and 8-methyl ThA added as indicated. The acetone powder extract containing 13 mg protein and the S. faecalis extract containing 0.2 mg protein were incubated at 32° C. for 90 min.

KCl concentration	Acetyl phosphate formed		Inhibition
	No inhibitor	4 mg 8-methyl ThA	
µM	µM	µM	%
None	1.06	0.52	51
40	1.07	0.62	42
50	1.10	0.84	24
70	1.13	0.98	13
100	1.23	1.21	0

antagonistic to ThA in the acetate activating reaction the 8-methyl compound must be converted to the dithiol form. This activation is apparently achieved by the same potassium sensitive system which activates ThA.

Jones, et. al. (6) demonstrated that the initial step in acetate activation is the formation of an enzyme-AMP complex (reaction 2). This may be measured by the rate of exchange between isotopic PP

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{PP} + \text{Enzyme-AMP} \qquad (2)$$

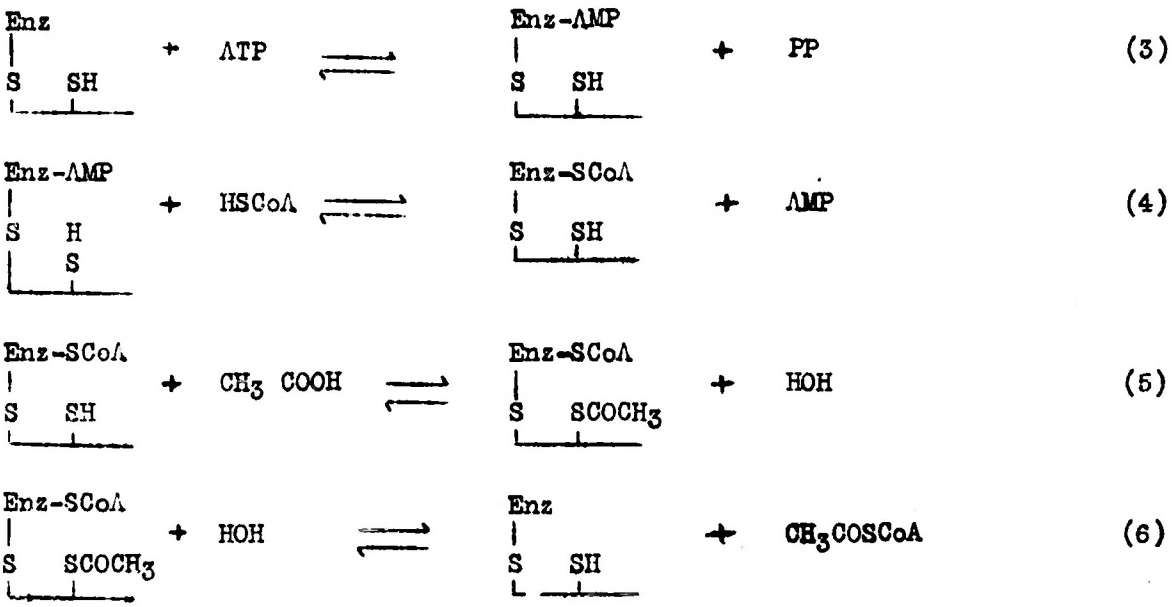
and ATP. Co A inhibits this exchange, indicating a subsequent exchange of Co A for AMP. Table IV shows that dithiol ThA also inhibits this pyrophosphate exchange; the disulphide compound is without effect.

TABLE IV. P^{32} Exchange between Inorganic PP and ATP

Incubation mixture contained in 1 ml: 3.4 µM P^{32} pyrophosphate, at pH 7.5; 2.5 µM K-ATP; 30 µM KF; 10 µM Mg Cl_2; 20 µM H_2S; 200 µM Tris buffer, pH 7.5; additions as listed. Incubated in air 20 min. at 37° C.

Additions	pyrophosphate		ATP		Exchange
	cts/min/ml.	cts/min/µM	cts/min/ml.	cts/min/µM	%
None	92,970	27,700	670	270	1.7
11 units enzyme	63,100	18,600	28,700	12,170	75.9
11 units enzyme + 2 µM Co A	76,610	23,520	16,580	7,080	42.2
11 units enzyme + 2 µM Co A	62,960	19,350	28,910	13,800	79.5
11 units enzyme + 2 µM dihydro ThA	76,340	25,210	15,020	6,730	38.6
11 units enzyme + 10 µM KCN	60,630	19,550	33,530	15,420	86.5
11 units enzyme + 2 µM dihydro ThA + 10 µM KCN	68,340	21,180	24,000	10,000	59.7

Cyanide accelerates the pyrophosphate exchange and when in combination with dithiol ThA, it reverses the dihydrothioctic acid inhibition. This is compatible with the proposal that in the acetate system, ThA is bound to the enzyme protein by a S-C linkage, which is readily broken by cyanide. Inhibition of the pyrophosphate exchange by dithiol ThA is then the result of f rmation of large amounts of this enzyme-dithiol thioctic acid complex which speeds reaction 2 to the right. The reaction sequence of acetate oxidation may then be visualized to proceed as:



It is possible that reactions 4 and 5 may be reversed in order; that is, that acetate may combine with the enzyme-thioctic complex prior to the exchange between Co A and AMP. The sequence of these changes are being investigated at present.

The Thioctic Acid Splitting Enzyme

The enzyme fraction of pigeon liver acetone powder extracts which is precipitated between 35-70% ammonium sulfate saturation contains most of the acetate activating activity of the extract. This fraction however does not respond to the alumina treatment for the removal of ThA. However, combination with the lower ammonium sulfate fraction (0-35% saturation) which contains only slight acetate activating activity, results in a mixture which responds to the alumina procedure

(Table V). The mixture of the two fractions responds to arsenite inhibitions as does the unfractionated extract (cf. Table II). It seems that the low ammonium sulfate fraction functions by splitting protein bound ThA; liberated ThA is then adsorbed on the alumina.

TABLE V. Alumina Treatment of Ammonium Sulfate Fractions of Pigeon Liver Acetone Powder Extracts

The incubation mixture was as described for system 3 in the legend of Table I. The ThA content of the 35-70% fraction amounted to 0.299 µg before alumina treatment. After treatment in combination with the 0-35% fraction, the cofactor content was 0.043 µg. Alumina treatment of the 35-70% fraction alone did not alter the ThA content. Incubations were for 90 min. at 37° C.

Additions and treatments	Citrate Formed		
	0-35% fraction (18 mg protein)	35-70% fraction (12 mg protein)	0-35% fraction (3 mg protein) + 35-70% fraction (12 mg protein)
	µM	µM	µM
None	0.03	0.86	0.84
+ 0.1 µg ThA	0.02	0.84	0.85
+ 4 µM arsenite	----	0.72	0.68
Alumina treated	----	0.88	0.13
+ 0.1 µg ThA	----	0.88	0.87
+ 0.1 µg ThA + 2 µM arsenite	----	----	0.31
+ 0.1 µg ThA + 2 µM arsenite + 5 µM BAL	----	----	0.82

It seems possible then that ThA can be removed from highly purified enzymes by the alumina procedure--in conjunction with the 0-35% ammonium sulfate fraction from pigeon liver extracts. The large amounts of cofactor in purified pyruvic oxidase (6) and α-ketoglutaric oxidase (7) are so tightly bound to the protein that such procedures as dialysis, repeated washing, or ion-exchange techniques do not dislodge it.

Table VI shows that the procedure is effective in removing ThA from Highly purified pyruvic oxidase from pigeon breast muscle. The almina procedure, also in conjunction with the splitting fraction (0-35% ammonium sulfate) from pigeon liver extracts, removes the cofactor from purified α-ketoglutaric oxidase (Fig. 4).

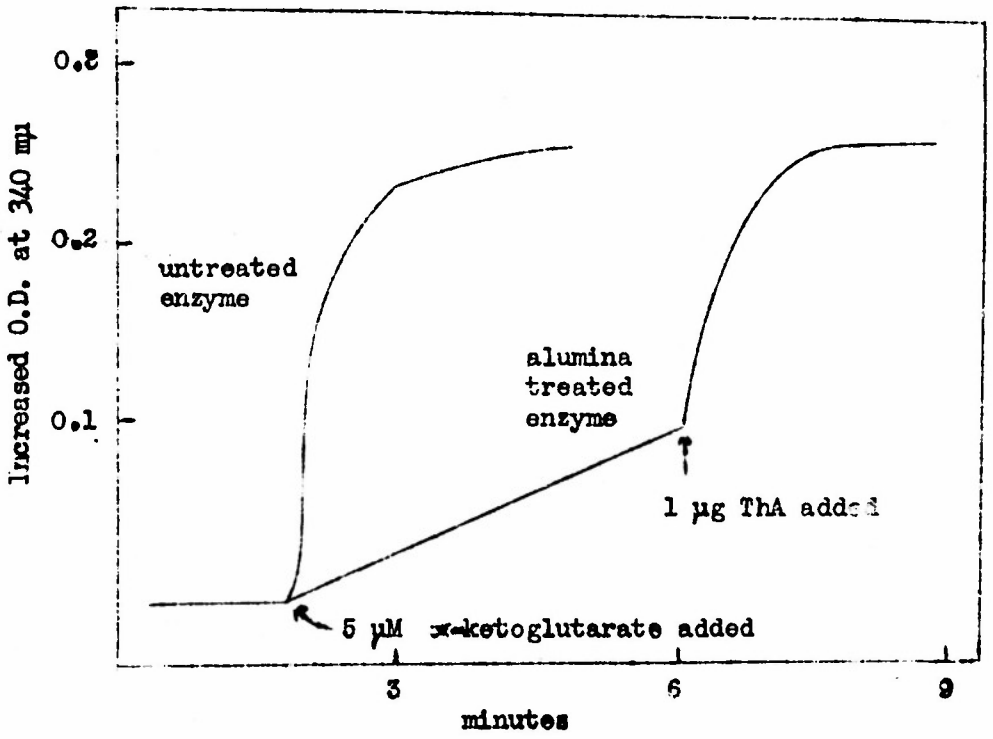
TABLE VI. Removal of ThA from Purified Pyruvic Oxidase

Incubation mixture contained in 1.0 ml: 50 µM phosphate buffer, pH 7.4; 2.4 µM $MgCl_2$; 1.6 µM $MnCl_2$; 1 µM GSH; 0.15 µM DPN; 0.47 µM DPT; 28 units CoA; 50 µM Na pyruvate, and 0.24 mg protein of S. faecalis extract. The pyruvic oxidase was of specific activity 42. The liver fraction was added in amounts containing 35 mg. of protein. Incubations were carried out for 60 min. under nitrogen at 38° C.

Enzyme mixture and treatment	ThA content of added enzyme mixture	Acetyl phosphate formed
	µg	µM
Pyruvic oxidase		
None	0.178	1.7
Alumina treated	0.183	1.7
Pyruvic oxidase + liver 0-35% fraction		
None	0.380	1.6
+ 1.0 µg ThA	-----	1.7
Alumina treated	0.044	0.2
+ 1.0 µg ThA	-----	1.8

FIGURE 4. Effect of Alumina Treatment of α-ketoglutaric Oxidase on Rate of Reduction of DPN

Incubation mixture contained in 3.0 ml: 75 units of Co A; 50 μM GSH; 0.3 μM DPN; 100 μM glycylglycine, pH 7.2, and enzyme mixture. The enzyme mixture contained 0.35 mg protein of α-ketoglutaric oxidase, specific activity 66, and 10.9 mg of 0-35% liver fraction. The ThA content of the untreated mixture was 0.263 μg, and in the alumina treated mixture the cofactor amounted to 0.016 μg.



III METHODS

Pigeon liver acetone powder extracted as described by Kaplan and Lipmann (8) served as the source of acetate activating enzyme. Insoluble materials were removed by centrifugation at 18,000 x g for 15 min. Co A was removed from the extracts by treatment with Dowex-1 (9). Pyruvic oxidase was purified from pigeon breast muscle according to Jagannathan and Schweet (10). Purification of α-ketoglutaric oxidase from pig heart followed the procedure of Sanadi, et al. (7).

Acetate activating activity was measured by either of three assay systems: (a) the acetyl-Co A formed by reaction 1 was non-enzymatically converted to acethydroxamate in the presence of high concentrations of hydroxylamine (reaction 2)(11); (b) inorganic phosphate served as acyl acceptor to form acetyl

$$\text{acetyl-Co A} + \text{hydroxlamine} \rightleftharpoons \text{CoA} + \text{acetylhydroxamate} \qquad (7)$$

phosphate in the presence of phosphotransacetylase (reaction3)(9); (c) oxaloacetate served as acceptor to form citrate (reaction 4)(12)(sufficient condensing enzyme is present in the extracts (13).

$$\text{acetyl-Co A} + \text{Pi} \underset{\text{phosphotransacetylase}}{\rightleftharpoons} \text{acetylphosphate} + \text{Co A} \qquad (8)$$

$$\text{acetyl-Co A} + \text{oxaloacetate} \underset{\text{condensing enzyme}}{\rightleftharpoons} \text{citrate} + \text{Co A} \qquad (9)$$

Pyruvate oxidation was measured by following the rate of dismutation to acetyl phosphate, CO_2 and lactate:

$$\text{pyruvate} + \text{DPN} + \text{Co A} \underset{\text{pyruvate oxidase}}{\rightleftharpoons} \text{acetyl-Co A} + CO_2 + \text{DPNH} \qquad (10)$$

$$\text{acetyl-CoA} + \text{Pi} \underset{\text{phosphotransacetylase}}{\rightleftharpoons} \text{acetylphosphate} + \text{Co A} \qquad (11)$$

$$\text{pyruvate} + \text{DPNH} \underset{\text{lactic dehydrogenase}}{\rightleftharpoons} \text{lactate} + \text{DPN} \qquad (12)$$

$$\text{SUM:}\quad 2\ \text{pyruvate} + \text{Pi} \rightleftharpoons \text{acetyl phosphate} + CO_2 + \text{lactate} \qquad (13)$$

The initial step in the oxidation of α-ketoglutarate (reaction 9) which is analogous to the oxidation of pyruvate (reaction 5), was measured by following

$$\alpha\text{-ketoglutarate} + \text{DPN} + \text{Co A} \rightleftharpoons \text{succinyl-Co A} + CO_2 + \text{DPNH} \qquad (14)$$

the reduction of DPN by the increase in optical density at 340 mμ in the Beckman DU spectrophotometer.

Acetyl phosphate and acethydroxamate were determined according to Lipmann and Tuttle (14, 15). Citrate was determined according to Natelson, et al. (16). An ammonium sulfate fraction of an extract of Streptococcus faecalis strain 10C1 which had been growing in media deficient in ThA (17) served as source of phosphotransacetylase and of lactic dehydrogenase.

Protein was determined turbidimetrically (9). The ThA content of enzymes was determined manometrically (17) following hydrolysis in 6 N H_2SO_4 at 120° C. for 1 hour.

Alumina treatments of enzymes were carried out in conical centrifuge tubes. The enzyme solution was brought to room temperature and was then rapidly stirred for 3 min. with adsorption alumina. The adsorbant was used in a ratio of 1 gram for each 100 mg of protein. The tube was then cooled in an ice bath and the alumina was allowed to settle. The supernatant fluid was pipetted off and was then centrifuged at 2000 x g for 15 min. at 4° C. to remove the remainder of the alumina. Treatment at room temperature appears to effect more complete splitting of ThA from the enzyme protein and adsorption than is accomplished at lower temperatures. Adsorption alumina with adsorptive capacity barely equivalent to a Brockman characterization of II is satisfactory. Large mesh (80-200) adsorbant is routinely used directly from the manufacturer's container. Acid or alkali washings do not uniformly affect the ability to adsorb ThA.

Isotopic exchanges between pyrophosphate and ATP were carried out with fraction 4 of the acetate enzyme from yeast (19), as described by Jones, et al. (6).

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V PUBLICATIONS RESULTING FROM GRANT

1. Participation of thioctic acid in the acetate-activating reaction. J. Amer. Chem. Soc., 76, 1712

2. Discussion on the requirement for thioctic acid in the acetate activating system. Presented at the "Symposium on B-Vitamins" sponsored by the Biochemical Institute of the University of Texas and the Clayton Foundation for Research, December 3, 1953, Austin, Texas.

3. Discussion on the coenzyme form in thioctic acid. Presented at symposium on "The Metabolic Role of Lipoic Acid (Thioctic Acid)" sponsored by the American Society of Biological Chemists, April 14, 1954, Atlantic City, N.J. Fed. Proc., 13, 731.

4. Pyruvate oxidation by extracts of Tetrahymena pyriformis. J. Gen. Microbiol., 11, 300.

5. Removal of thioctic acid from enzymes. J. Biol. Chem., in press.

6. Thioctic acid in the animal acetate-activating reaction, in preparation.